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Abbreviations: HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HR, homologous recombination; HBVHCC, HBV-associated hepatocarcinoma; DSB, DNA double strand break; PHHs, primary human hepatocytes; CPT, camptothecin; IRIF, ionising radiation-induced foci; ChIP, chromatin immunoprecipitation; DAPI, 4,6-diamidino-2-pheny-lindole.

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None.

Author contributions

L.R., M.Z. and Z.T. proved the interference of HBx and function of $CRL4^{WDR70}$ in HR and contributed to data interpretation. M.Z. set up DSB repair and ChIP assays. X.J.W., Y.X., H.W., X.B.W., Y.W., X.W., L.G., B.Z. and D.M supported virology and cellular studies supervised by M.L., N.Z. and X.H.W. provided insightful analysis for WDR70 structural analysis. J.H advised on histone modifications. L.R. performed PHH verification assays. L.R., S.W. and J.L. analysed human samples. A.M.C. and P.J. advised on DDR assay. C.L. supervised the overall project and wrote the manuscript. A.M.C edited the manuscript.

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Abstract

Chronic infection of hepatitis virus B (HBV) is associated with an increased incidence of hepatocellular carcinoma (HCC). HBV encodes an oncoprotein (HBx) that is crucial for viral replication and interferes with multiple cellular activities including gene expression, histone modifications and genomic stability. To date, it remains unclear how disruption of these activities contributes to hepatocarcinogenesis. Here, we report that HBV exhibits a novel anti-resection activity by disrupting DNA end resection, thus impairing the initial steps of homologous recombination (HR). This anti-resection activity occurs in primary human hepatocytes (PHHs) undergoing a natural viral infection-replication cycle, as well as in cells

with integrated HBV genomes. Among the seven HBV-encoded proteins, we identified HBx as the sole viral factor that inhibits resection. By disrupting an evolutionarily conserved Cullin4A-DDB1-RING type of E3 ligase, $CRL4^{WDR70}$, via its H-box, we show that HBx inhibits H2B monoubiquitylation at lysine 120 (uH2B) at double strand breaks, thus reducing the efficiency of long-range resection. We further show that directly impairing H2B monoubiquitylation elicited tumorigenesis upon engraftment of deficient cells in athymic mice, confirming that the impairment of $CRL4^{WDR70}$ function by HBx is sufficient to promote carcinogenesis. Finally, we demonstrated that lack of H2B monoubiquitylation is manifest in human HBV-associated HCC (HBVHCC) when compared with HBV-free HCC, implying corresponding defects of epigenetic regulation and end resection. We conclude that the anti-resection activity of HBx induces an HR defect and genome instability and contributes to tumorigenesis of host hepatocytes.

Introduction

Worldwide, chronic HBV infection and its associated diseases such as cirrhosis account for about 50% of all cases of human hepatocellular carcinoma (HCC) and the risk of carcinogenesis is elevated with increasing viral load (1, 2). The vast majority of the HBV-associated hepatocellular carcinomas (HBVHCC) harbour chromosomally integrated HBV genomes (3, 4), although site-specific integration of the viral genome into known oncogene seems to be a rare event. This strongly suggests that HBV encodes a viral element that intrinsically promotes oncogenic transformation. During HBV infection, the small 3.2-Kb HBV genome expresses seven genes (preCore (HBeAg), Core, Pol, X, and three envelop proteins, L, M and S) for completion of the viral replication (5). The *X* gene encoding a 154-residue pleiotropic transcriptional activator (HBx) serves as a stimulator of viral replication and gene expression (6, 7). A plethora of evidence suggests that HBx has the capacity to induce HCC *in vivo*, behaving as a prominent oncogenic driver for HBVHCC (8). However, the precise oncogenic targets and/or signal transduction pathways influenced by HBx remain enigmatic.

HBx lacks DNA binding activity and thus likely carries out its functions via protein-protein interactions with cellular factors. One such HBx-interacting factor is DDB1, a core component of the Cullin-4 ring ligase (CRL4) family of ubiquitin ligases (9). The core of CRL4 complexes (CUL4-DDB1-ROC) is associated with a family of WD40 proteins, which act as substrate-targeting subunits and are known as DCAFs (10). Two short peptide stretches of DCAFs, the DWD (DDB1-binding WD40) and H-box motifs, are required for the docking of DCAFs to CRL4s via DDB1 (11-13). Intriguingly, the C-terminus of HBx resembles the H-box of a subset of cellular DCAFs (including DDB2 and DCAF9/WDTC1) and can selectively disengage DCAFs from CRL4 (12, 14). Recent studies have shown that HBx hijacks the E3 ligase activity of CRL4 to form viral $CRL4^{HBx}$ and remove specific cellular factors (i.e. SMC5/6) that are restrictive to viral replication (15).

Impairment of the CRL4 core complex activitiy by tissue-specific knockout of *DDB1* in hepatocytes has been shown to induce liver regeneration and tumorigenesis (16), suggesting a restriction of HCC by CRL4. This could be attributed to the potential of HBx to interfere with DNA damage responses: it compromises cell cycle checkpoint control by its interaction with p53 (9) and inhibits the UV light-induced nucleotide excision repair (NER) pathway by disrupting the $CRL4^{DDB2}$ complex (17). However, no role has been established for disabled NER in promoting human HBVHCC and removal of known DCAF factors do not account for the oncogenic activity of HBx. Thus, there exists a mechanistic gap in HBx-triggered hepatocarcinogenesis, implying an uncharacterised mechanism underlying HBVHCC.

Here, we present evidence that HBV infection inhibits DNA double strand break (DSB) repair in both HBV infected and HBV-integrated cells. We show that the disassembly of a recently identified CRL4 sub-complex, $CRL4^{WDR70}$, by the HBx H-box motif compromises DNA end resection. The resulting loss of $CRL4^{WDR70}$-dependent histone H2B monoubiquitylation prevents the association of RPA and RAD51 with DSBs and prevents efficient homologous recombination (HR) of DSBs. We demonstrate that the loss of WDR70

function phenocopies the impact of HBx on HR and establish that the epigenetic and repair defects caused by HBx can directly trigger tumorigenesis. Thus, this study provides compelling evidence that HBx possesses an anti-resection activity that inhibits $CRL4^{WDR70}$-mediated HR repair. These results in genome instability of host cells and potentially drives the carcinogenesis of HBVHCC.

Experimental Procedures

Cell lines

L02, T43 (18), HepG2, HepG2.2.15, HEK293T and their derived cells were cultured in standard conditions. PHHs culture and infection with HBV were performed as described previously (19, 20). Additional details are in Supporting Materials.

Biochemical, immunological and molecular assays

Details of immunoassys, measurement of DNA damage responses, (RT-)qPCR and ChIP analysis are available in the supporting methods.

Statistics

All histograms were presented as means ± standard deviation. For quantitative analysis, including the ChIP assay and image analysis, at least three independent experiments each containing three parallel samples were performed. Statistical analysis between two groups was performed by *t*-test in GraphPad Prism 5: *, $p<0.05$; **, p, 0.01; ***, $p<0.001$; NS, no statistical significance.

Results

HBV infection induces a resection defect in host cells

HBVHCC genomes are remarkably unstable ((21) and data not shown). We therefore examined whether HBV impacts on DSB repair, the malfunction of which often causes genome-wide structural variations. Congenic hepatocarcinoma cell lines (parental HepG2 and derived HepG2.2.15 that harbours a functional HBV genome (22)) were challenged with DSB-generating agents and DSB responses were evaluation by indirect immunofluorescence. Upon camptothecin (CPT) treatment HepG2.2.15 cells, when

compared to control HepG2, showed reduced foci formation for NBS1 and RPA32, the latter of which is a replication protein A (RPA) subunit. RPA is a prominent marker for the ssDNA that arises from 5'-3' DSB resection (23) (Supporting Fig.S1A). Phosphorylation of RPA32 (pSerine 33 and pSerine 4/8) was also shown to be diminished by immunoblotting (Fig.1A and Supporting Fig.S1B). Since RPA constitutes a platform to attract HR factors onto processed DNA ends and is a prerequisite for HR (see Supporting Fig. S1C), these results suggest that DNA end resection, and consequently HR itself is compromised (24).

To confirm that DSB resection is attenuated in HepG2.2.15 cells, single stranded DNA was visualised directly using BrdU incorporation and subsequent α-BrdU antibody staining under non-denaturing conditions (Fig.1B). Because the generation of sufficient ssDNA from DNA end resection is required for the recruitment of Rad51 recombinase to mediate homology search and downstream HR events (25), we next visualised RAD51 foci. In HepG2.2.15 cells, the recruitment of RAD51 to DSBs after ionising radiation (IR) was dramatically decreased in comparison with HepG2 controls (Fig.1C). Thus, HBV is associated with resection and HR defects in a hepatocarcinoma cell line with integrated with viral genomes.

Resection defect in HBV-infected cells

Although HepG2.2.15 is a widely used as a model system for studying HBV replication, the hepatocarcinoma origin of this cell line and random integration of the viral genome may result in genetic variation of the host genome and therefore unfaithfully represent the cellular impact of HBV. To exclude this possibility and extend our observations to the circumstances of natural viral infection, we present two lines of evidence to establish that the HBV-induced resection and HR defects represent a generalised phenomenon. First, we show that the defects in RPA phosphorylation and RAD51 IRIF (ionising radiation-induced foci) hold true in an independently derived HBV-positive cell line (T43) that was generated by integrating viral genomes into normal hepatic L02 cells (18) (Fig.1D, F and Supporting Fig.S1D).

Second, we employed HBV infected primary hepatocyte cultures (PHHs) to investigate the impact of natural viral infection on resection activity (19, 20). The application of concentrated supernatants from HepG2.2.15 cells, or high-titre HBV patient serum, to PHHs resulted in efficient infection and viral replication as shown by increased HBsAg/HBeAg titres and the positivity for nucleocapsid antigen (HBcAg) during day 3-7 post-inoculation (Supporting Fig.S1E-F). As a consequence of HBV infection, IR-induced RPA32 phosphorylation was reduced significantly in PHHs, coincidental with reduced RPA32/RAD51 IRIF in HBcAg-positive cells (Fig.1E-F). Taken together, these data demonstrate that DNA end resection is impeded during the course of HBV infection and maintained after integration of viral genomes.

HBx counteracts CUL4A-DDB1-dependent resection via its H-box motif

Genes involved in homology-dependent repair are frequently mutated in breast and ovarian cancer (26, 27). However, no human genetic abnormalities involving HR have been described for HBVHCC. To consolidate our observation that HBV-induced HR defects are directly attributable to a viral element, as opposed to genetic variation in the host genomes, we performed a screen for the impact of HBV viral genes on resection (Supporting Fig.S2A). By examining CPT-induced RPA and NBS1 phosphorylation, HBx was identified as the sole viral factor that renders cells defective in resection (Fig.2A and Supporting Fig.2B). HBx expression in L02 cells also perturbed RPA32 and RAD51 foci formation (Fig.2B and Supporting Fig.S2C). In corresponding experiments, we also showed that the defects in RPA phosphorylation and DSB loading were significantly restored in T43 cells upon silencing of *HBx* by siRNA (Fig.2C and Supporting Fig.S2D-E). *siHBx* could also rescue the defective IRIF of RPA and RAD51 in PHH cells (Supporting Fig.S2F).

To exclude the possibility that overexpression of *HBx* produces off-target effects not seen in physiological conditions, mRNA levels of *HBx* in T43, HepG2.2.15 and PHHs were quantified (Supporting Fig.2G). T43 cells express *HBx* at a comparable level to infected PHHs, but at significantly lower levels than that observed in HepG2.2.15. This likely reflects

the relative replicative activities, as indicated by the cccDNA copies (Supporting Fig.2H). Thus, we conclude that HBx confers HBV an anti-resection activity under physiological conditions.

The majority of HBx protein is in a complex with DDB1 that is mediated by its H-box motif (residues 88-98) (28). This results in the re-direction of the CRL4 activity to support the viral life cycle (15, 29). We therefore tested the hypothesis that HBx interferes with resection by disrupting the function of CRL4 complexes. Indeed, the formation of RPA32 and RAD51 IRIF was diminished upon *DDB1* depletion by RNAi technology, indicating that a species of CRL4 complex that participates in regulating the formation of ssDNA filaments and resection is downregulated (Supporting Fig.S3A). CPT-induced phosphorylation of RPA32 / NBS1 upon si*DDB1* or si*CUL4A* treatment was also affected, resembling the phenotype of HBx expression (Supporting Fig.S3B-C). The similar HR defects caused by *CRL4* ablation and HBx introduction suggest a possible mechanistic link between these factors in affecting DNA end resection.

To elucidate how HBx impairs the resection process by interfering with CUL4A-DDB1, various mutants encompassing the H-box motif and the HBx carboxyl domain were constructed (Supporting Fig.S3D). Ectopic expression of these *HBx* mutants near or within the H-box (*d3*:C105-154Δ, *Hdel*: C88-100Δ and R96E) abolished the HBx-DDB1 association (Supporting Fig.S3E-G), consistent with a previous report (12). Interestingly, upon ectopic expression in L02 cells, abolition of the H-box impaired the anti-resection potential of HBx and the mutants displayed no profound impact on RPA32 phosphorylation or foci formation in contrast to *HBx* wild type (Fig. 2D-F). Therefore, we conclude that HBx via its H-box targets the function of CRL4 complex to impede DNA end resection.

HBx interferes with CRL4^{WDR70}-mediated DNA end resection

To identify the CRL4 complex targeted by HBx in respect of end resection we examined the recently identified CRL4^{WDR70} complex that regulates resection in fission yeast (30). Similar

to the yeast homologue, WDR70 interacts with DDB1 (Supporting Fig.S4A). WDR70 protein contains a canonical DWD motif (residues 405-408 AA in the human homologue) as well as a conserved C-terminal H-box (653-662 AA) (Fig.3A-B). Both domains are indispensable in mediating the interaction of WDR70 with DDB1 (Fig.3C, (12, 30)). Strikingly, the C-terminus of WDR70 contains a similar feature to that characterised for DDB2 sequence: an additional α-helix adjunct to H-box that provides additional binding capacity between DDB1 and DDB2. The presence of this conserved additional α-helix is predicated to provide extra binding capabilities for WDR70 and DDB1 (Fig.3B and Supporting Fig.S4B).

The structural similarities between WDR70 and DDB2 suggest that WDR70 resembles the subclass of DCAF proteins (including DDB2) whose engagement to DDB1 via their H-box is liable to competition by the homologous sequence of HBx (28). This would be consistent with the fact that knockout of human *WDR70* by CRISPR in 293T cells (30) resulted in decreased NBS1/RPA32 phosphorylation and diminished RAD51 recruitment. If HBx is influencing HR mainly through an interaction with WDR70, we would predict that expressing HBx in *WDR70* knockout cells would not further decrease NBS1 or RAP32 phosphorylation. Indeed, when HBx was expressed in *WDR70* knockout cells, there was no further decrease in NBS1 or RPA32 phosphorylation (Supporting Fig.S4C) following IR treatment and RPA plus RAD51 foci formation were also not decreased further (Fig.3D-E). This strongly suggests that the anti-resection activity of HBx can primarily be attributed to impairment of $CRL4^{WDR70}$ function.

DSB repair is mainly mediated by two pathways, non-homologous end joining (NHEJ) and homologous recombination. NHEJ is active throughout the cell cycle while HR is restricted to the S/G2 phases due to the requirement for sister chromatid template (31). Therefore, a reduction of the S/G2 population could indirectly influence the observed resection efficiency without impairing HR machineries. However, FACS analysis showed only a modest <20% increase of G1 phase cells (with a corresponding decrease of S/G2 phase) in *WDR70* or *DDB1* ablated cells. This could not account for the >50% reduction observed in IRIF and immunoblotting assays (Supporting Fig.S4D). Thus, the malfunction of

HR observed upon loss of *WDR70* is primarily caused by inhibited end processing *per se* and not by cell cycle perturbation. We confirmed this was also the case in HBV containing cells by distinguishing G1 and S/G2 populations of T43 and HepG2.2.15 using the FUCCI system (Fluorescent ubiquitylation-based cell cycle indicator (32)). No apparent perturbation to the cell cycle distributions were observed (data not shown). Moreover, in *WDR70* knockout, T43 and *HBx* expressing cells, the reduction of RAD51/RPA32 IRIF was only apparent in Geminin-positive cells. Geminin staining specifically marks S/G2 cells (Fig.3F-G). Thus, HBx interferes with the $CRL4^{WDR70}$ complex and suppresses resection in S/G2.

HBx inhibits resection by acting as a dominant negative for the assembly of $CRL4^{WDR70}$

To test our hypothesis that HBx can engage with DDB1 via its H-box and consequently displaces WDR70 from the CRL4 scaffold we examine the impact of *HBx* on $CRL4^{WDR70}$ complex integrity. As predicted, the binding of WDR70 to DDB1 was effectively competed by *HBx* expressed from either lentivirus (Fig.4A) or at low level expression from integrated HBV genomes (T43) (Fig.4B and Supporting Fig.S5A-B). The attenuation of the DDB1-WDR70 interaction in T43 cells could be effectively reversed by ablating HBx with siRNA (Fig.4C). Moreover, both *DDB1* or *WDR70* over-expression overrode the reduction of RPA32 phosphorylation in T43 and *HBx*-expressing L02 cells (Fig.4D). These data strongly indicate HBx, even at relatively low levels of expression, disrupts the formation of the $CRL4^{WDR70}$ complex.

It is likely that the HR defect observed when HBx is expressed results from loss of the $CRL4^{WDR70}$ complex, since we have shown that expressing HBx in *WDR70* knockout cells did not exacerbate the HR phenotype (Fig 3D,E). None the less, it is possible that, because HBx forms a viral CRL4 complex and re-directs the ubiquitin ligase activity to decrease the levels of specific cellular proteins (15), HBx could induce HR defects directly, for example by degrading a key HR protein (11, 33). To further address this possibility, SV5-V, a protein encoded by a paramyxovirus (Simian virus 5) that shares a homologous H-box with HBx (33) (Supporting Fig.S5C), was employed.

Apart from their similar ability to bind DDB1, the amino acid sequence and functions of SV5 V protein and HBx are unique and cannot substitute for each other in terms of viral-host interaction. We reasoned that, if HBx forms a novel CRL4 E3 ligase to target a HR protein, SV5 V protein will not be able to substitute for HBx in terms of its inhibition of resection. Interestingly, we observed a clear impact of SV5 V protein on RPA phosphorylation (Fig.4E) and on the assembly of the of $CRL4^{WDR70}$ complex, equivalent to that seen for HBx (Fig.4F). This corroborates that viral H-boxes, regardless of their origin, are sufficient to disrupt $CRL4^{WDR70}$-dependent resection. It also strongly argues against the possibility that HBx reconstitutes the CRL4 ubiquitin ligase activity to target a repair protein that is key for resection. We therefore conclude that HBx directly blocks the assembly of $CRL4^{WDR70}$ and that this, rather than the reprogramming the CRL4 activity, is the cause of the HR defects we observe.

Reduction of $CRL4^{WDR70}$-dependent H2B monoubiquitylation in the presence of HBx

DNA lesions are repaired in the context of chromatin and the compact nucleosomal structure needs to be remodelled to allow damaged DNA to become accessible to repair factors (34-36). Give the role of yeast $CRL4^{Wdr70}$ in coordinating DSB repair and histone modification (30), we investigated a variety of histone modifications under the condition of *HBx* expression and $CRL4^{WDR70}$ depletion and in HepG2.2.15 cells. Notably, levels of H2B monoubiquitylation (K120) and multiple forms of methylated H3K4 and H3K9 were reduced in when HBx was present (Fig.5A-C and Supporting Fig.S6A). Again, natural HBV infection in PHHs and ectopic expression of SV5-V also strongly decreased H2B modification at K120 (Fig.5D and Supporting Fig.S6B).

H2B monoubiquitylation is required for the timely repair of DSBs and malfunction of this pathway is implicated in tumorigenesis (37, 38). Similar to the impact of *HBx*-expression and *WDR70* depletion, the introduction of non-ubiquitylatable H2B (*2KR*: K120R/K125R) into L02 cells prevented RPA32 chromatin association (Fig.5E). This is consistent with H2B monoubiquitylation being required for resection. We could again link this defect directly to

CRL4^{WDR70} since the deficiency in H2B monoubiquitylation observed in T43 and HBx expressing L02 cells could be complemented by *WDR70* expression, congruent with the rescue of RPA phosphorylation (Fig.5F and Supporting Fig.S6C). In addition, ablation of *HBx* by siRNA in T43 cells also restored the level of uH2B (Fig.S6D). Furthermore, ablating the components of HULC^{RNF20}, the ubiquitin ligase for H2B monoubiquitylation (39), inhibited RPA phosphorylation as well as the foci formation of RPA32 and RAD51, which again displayed epistasis with *WDR70* depletion (Supporting Fig.S6E-G). These results consolidate the notion that the anti-HR effect of HBx is exerted through chromatin remodeling events co-regulated by HULC^{RNF20} and CRL4^{WDR70}.

HBx impedes extensive H2B monoubiquitylation and long-range resection

To demonstrate that the perturbation of CRL4^{WDR70}-dependent H2B monoubiquitylation by HBx occurs at the chromatin level, we employed ChIP assays (chromatin immunoprecipitation) to characterise the chromatin dynamics at *Intron 1* of the *PPP1R12C/p84* gene (Fig.6A). When DSBs were artificially induced by CRISPR-gRNA at this locus, the enrichment of H2B monoubiquitylation at regions surrounding an induced DSBs was weakened upon *WDR70* knockout or in the presence of HBV/HBx (Fig.6B). Intriguingly, the presence of HBx or the loss of *WDR70* markedly impaired the enrichment of H2B monoubiquitylation at regions distal to DNA breaks (>3.5 Kb) but exerted no detectable impacts in the proximal regions tested (0.5 Kb and 3.5 Kb). In concordance with the impact of HBx on CRL4^{WDR70} complex integrity, this regional effect was correlated with the ability of HBx to exclude the loading of DDB1 and WDR70 from distal chromatin domains of DSBs (data not shown). Thus, HBx disables an evolutionarily conserved role of CRL4^{WDR70} in expanding the H2B monoubiquitylation towards DSB-distal regions.

The progression of resection is catalysed by the cooperation of several enzymes: CtIP and the MRN nuclease initiate resection to generate a short ssDNA substrate. This is subsequently extended by EXO1 and BLM^{Rqh1}-DNA2, channeling repair into the HR pathways (40). To dissect how HBx influences the chromatin loading of resection factors via down-regulation of H2B monoubiquitylation, patterns of chromatin recruitment for a variety of

repair factors were analysed by ChIP assays using the CRISPR-induced DSB assay. Strikingly, we detected a reduction of RPA32 recruitment at 10 Kb and 50 Kb from the DSB site in the presence of HBx or following the loss of *WDR70* (Fig.6C). This suggests that the failed extension of H2B modification to the DSB-distal prevents long-range resection. Indeed, introduction of a non-ubiquitylatable *2KR* mutant of H2B into L02 cells reduced the RPA32 loading, replicating the defect caused by HBx and *WDR70* depletion (Fig.6D). Consistent to the resection defects monitored by RPA loading, the chromatin association of the long-range resection nuclease (EXO1) displayed a dramatic decrease exclusively at sites distal to the DSB upon depletion of *WDR70* or *HBx* expression. No alteration of DSB-enrichment of the resection initiator (MRE11) was detected in the same set of experiment (Fig.6E-F). Therefore, HBx induces $CRL4^{WDR70}$ disruption and this results in a reduction of H2B monoubiquitylation and specifically prevents the chromatin loading of EXO1 and the ensuing extensive resection.

Defective H2B monoubiquitylation promotes tumorigenesis

We have shown that HBx ablates the $CRL4^{WDR70}$ complex and that this results in a resection defect. The first observed event we see when $CRL4^{WDR70}$ is defective is a failure of H2B monoubiquitylation. To investigate if $CRL4^{WDR70}$-dependent H2B monoubiquitylation could contribute to tumour suppression, a stable cell line of L02 integrated with a lenti-viral *2KR* construct was established. Two million cells were then injected subcutaneously into immune defective athymic nude mice and tumour growth was monitored. The *2KR*-expressing L02 cells exhibited uncontrolled neoplastic proliferation when compared with control cells. Remarkably, co-expression of *WDR70* and *DDB1* genes suppressed this increased tumorigenesis (Fig.7A). Consistent with H2B monoubiquitylation acting in a tumour suppressor pathway, over-expression of the de-ubiquitinase USP22 (which eliminates H2B monoubiquitylation), also significantly promoted tumour growth (Fig.7B).

We also generated HBV-positive xenograft models by embedding L02 and T43 cells in nude mice. Resembling the *2KR* inoculums, T43 but not L02 inoculums resulted in aggressive tumour outgrowth (Fig.7C) that could be significantly repressed by

overexpression of *DDB1* or *WDR70* or by *in vivo* knockdown with *siHBx* (Fig.7D and Supporting Figure S7A). Examination of these tumours by immunohistochemical staining revealed that H2B monoubiquitylation was absent in T43-derived xenografts (Fig.7E). Taken together, these data support the idea that the $CRL4^{WDR70}$-H2B signalling pathway acts as a crucial anti-cancer mechanism, inhibition of which by HBx potentially promotes tumorigenesis.

Implication of the HBx-mediated anti-resection activity in human HBVHCC

As HBx is expressed in significant proportion of human HBVHCC, H2B monoubiquitylation, whose levels are correlated to the function of $CRL4^{WDR70}$-mediated resection, could serve as the predictive marker of HR status. We therefore examined H2B monoubiquiination in clinically isolated liver cancer tissues. Paraffin-embedded sections of cohorts of either HBV-positive or HBV-negative-HCC biopsies were subjected to immunohistochemical (IHC) staining with antibody specific to ubiquityl H2B (K120) (Fig.8A). In comparison to HCC samples that were negative for serological HBV surface antigen (HBs), sections from HBVHCC patients displayed a statistically lower intensity and percentage of uH2B when visualized by staining of α-uH2B (Fig.8B). A small proportion of HBVHCC displayed modest level of H2B monoubiquitylation, possibly due to occult infection (41) or an acquired second mutation such as 53BP1, which can potentially rescue HBx-induced HR defect. Similarly, uH2B levels in HBV-negative non-HCC tissues are also significantly higher than those of HBV-positive (Fig.8B and Supporting Fig.S7B). Thus, given the mechanistic link between HBx and $CRL4^{WDR70}$-mediated H2B monoubiquitylation, a reduction of uH2B levels in the majority of HBVHCC tissues can be potentially correlated with the anti-resection-activity of HBx.

Genetic variants of the *HBx* gene, such as missense mutations at residuals 5, 130 and 131, emerge during the course of HBV-associated liver diseases and finally prevail in late-stage HCC (42). These mutant forms, especial those locating in the C-terminus of HBx (130 and 131), could potentially disable the capability of HBx to block end resection. However, we found these clinically prevalent *HBx* mutants largely preserved the suppressive effect on

resection: none of these variants reduced the ability of HBx to suppress H2B monoubiquitylation or RPA32 and NBS1 phosphorylation (Fig.8C). This result correlates with the IHC assays that demonstrated diminished H2B monoubiquitylation in the majority of HBVHCC biopsies (Fig. 8A). Thus, despite the prevalence of mutations in late stages of HBVHCC, HBx maintains the potential to influence epigenetic regulation and end resection.

Discussion

HBx is the oncoprotein of HBV and is tightly linked to the progression of HBVHCC. HBx transcripts and protein can be detected in hepatocytes from 70% of chronically infected patients and in many HBVHCC biopsies (43, 44). However, how HBV and HBx promote liver cancer is not adequately understood. Here, we report for the first time that HBV impedes an early step of homologous recombination. We demonstrate that, via the HBx protein, HBV interferes with DNA end resection and HR repair. This occurs during both phases of acute infection (PHHs) and integration in the host genome (HepaG2.2.15 and T43). These data demonstrate that the HBx-mediated anti-resection activity is a genuine viral impact on host cells, as opposed to an artificial phenomena caused by random viral integration. Equally importantly, our analysis establishes that the integration of HBV fragments can cause long-term effect on genome stability: as long as viral replication is active, or the *HBx* is expressed (even at relatively low levels) during any stage of the disease (chronic infection, cirrhosis and hepatocarcinoma), resection and thus HR would be restrained. This can potentially render host genomes genetically unstable over extended periods of time (Fig.8D).

HBx disassembles specific CRL4 sub-complexes or retargets the CRL4 to support the viral life cycle (12, 15). A subset of DCAFs, including WDR70, is strongly dissociated from DDB1 in the presence of HBx. Our sequence alignment of the H-boxes of HBx and several DCAFs identified residues important for the DDB1 interaction (Fig.3A). Interestingly, WDR70 and DDB2 share C-terminal sequences that fold into successive α-helices and provide additional surfaces that bind to DDB1 (Fig.3B and Fig.S4B). Upon direct competition from

HBx, this additional binding is likely to be disrupted, leading to the dissociation of WDR70 from the CRL4 scaffold.

Our data establishes a functional link between HBx and CRL4-mediated DNA end resection based on the competition between HBx and WDR70 for DDB1 binding. We identified that it is the ablation of the $CRL4^{WDR70}$ subcomplex that is responsible for the HR defect in HBV-infected or HBV-integrated cells. We can rule out the possibility that HBx causes the HR defect through hijacking and reprogramming the ubiquitin ligase activity of CRL4s towards proteins required for resection for two reasons. First, expression of HBx does not further decrease the resection defects observed in *WDR70* knock out cells. Second, the anti-resection activity of HBx is entirely dependent on its H-box motif and the introduction of the SV5 H-box peptide is fully capable of disrupting $CRL4^{WDR70}$ complex integrity and subsequent HR events. These data directly link HBV-mediated HR interference to the structural integrity of CRL4 complexes. Therefore, impact of HBx on DDB1 results in two major HBV-related pathologies: by forming a viral $CRL4^{HBx}$ ubiquitin ligase, HBx promotes viral replication by targeting SMC5/6 for proteasome-dependent ubiqutiniation (45). In this case, CRL4 ubiquitination activity is obligatory for viral replication. Independent of $CRL4^{HBx}$ activities, HBx also exerts an anti-resection activity by blocking $CRL4^{WDR70}$ complex formation, which causes genome instability in host cells (data not shown). The complex influence of HBx on the CUL4-DDB1-related complexes shed lights on future drug design: for example. small molecules could be identified as anti-viral compounds, which prevent the DDB1-HBx interaction and/or restore the integrity of $CRL4^{WDR70}$.

In the context of HBV induced carcinogenesis, we show that loss of $CRL4^{WDR70}$ phenocopies HBV infection and HBx expression and has the potential to drive the oncogenic transformation by promoting genome instability. Ablation of *WDR70* caused an equivalent decrease of uH2B and DSB-recruitment of RPA/RAD51 as is seen during HBV infection and post-integration stage. Resembling the well-established model of breast cancer inflicted by *BRCA1* mutation (46, 47), a reduction of HR upon HBV infection predictes a threat to genome stability and render host cells susceptible to oncogenic transformation. We further

support the prediction that HBV promotes carcinogenesis by interfering with $CRL4^{WDR70}$ by showing that the ablation of H2B ubiquitylation (by expressing a *2KR* mutant) dramatically promoted oncogenic proliferation and that this could be effectively suppressed by concomitant over-expression of *WDR70*. Again this correlates well with the observation that the aggressive growth driven by HBV in T43 cells was accompanied by low level of uH2B in comparison to control cells. Therefore we conclude that the $CRL4^{WDR70}$-uH2B regulatory network is a novel anti-cancer mechanism and meets the criteria being a direct oncogenic target of HBx.

HBx is the crucial factor for HBV replication and antigen expression. This poses the question as to whether the modulation of resection benefits the viral replication. In previous work we showed that ablating of DDB1 inhibited viral production and gene expression of HBV, indicating that DDB1 is required for viral production (18). Our preliminary data also suggest that loss of *WDR70* facilitates viral replication (data not shown). There could be several putative mechanisms by which $CRL4^{WDR70}$ and H2B monoubiquitylation influence HBV replication: aberrant DNA structures (i.e. hairpins or single-stranded DNA) are formed during HBV replication and anti-viral mechanisms are likely to be activated in response to these potential signatures of viral infection. In such a scenario, a reduction of $CRL4^{WDR70}$-uH2B activity may be advantageous to curb unwanted processing of intermediate structures by host nuclease (such as BLM and EXO1). Alternatively, it is likely that $CRL4^{WDR70}$ is involved in regulating viral gene expression through modulating the levels of H2B monoubiquitylation in promoter regions (data not shown). Therefore, from the perspective of viral replication, HBx may facilitate viral gene expression and protect viral genomes by influencing histone modifications, transcription and host nucleases.

Taken together, our findings reveal that HBx interferes with the $CRL4^{WDR70}$-mediated DNA end resection and causes HR deficiency in HBV-infected hepatocytes. Impaired DSB repair contributes to genome instability and drive hepatocarcinogenesis.

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Figures legends

Fig.1. HBV attenuates DNA end resection upon genotoxic insults. (A) Immunoblotting for RPA32 phosphorylation in HepG2 and HepG2.2.15 cells upon CPT treatment. pRPA32: phosphorylated Serine33 of RPA32. Asterisk: band shift of phosphorylated RPA32 detected by α-RPA32 serum. (B) Non-(-HCl) and denaturing-(+HCl) indirect immunofluorescence (IF) showing ssDNA at resected DSBs and equal incorporation of BrdU after CPT treatment, respectively. Scale bar: 40 microns. (C) Representative image and percentage of RAD51 IRIF-positive cells after 5-Gy irradiation in HepG2 and HepG2.2.15 cells. (D) Representative images for IRIF of RAD51and phosphorylated RPA32 (pSerine 33) in L02 and T43 cells after IR treatment. (E) Images (top) and quantification (bottom) of indicated IRIF in mock or HBV-infected PHHs at day 7. Cells were fixed for immunostaining 4 hours after 1 Gy irradiation. HBcAg (green) marks HBV-infected PHHs. (F) Immunoblotting for RPA32 phosphorylation in L02/T43 cells or HBV-infected PHHs at indicated times. genotoxic treatments: 2 uM CPT or 5 Gy irradiation.

Fig.2. HBx impairs DNA end resection. (A) Immunoblotting for IR or CPT-induced RPA32 and NBS1 phosphorylation in the presence, or not, of HBx. L02 cells were pre-infected with 2 MOI/cell of pLV (mock infection) or pLV-HA-HBx lentivirus. (B) pLV or pLV-HBx transfected L02 cells were treated as in A, followed by immunostaining of RAD51 and pRPA32 foci. (C) Monitoring of RPA32 phosphorylation in indicated cells treated, or not, with *siHBX*. (D) Immunoblotting for pRPA32 in CPT-treated L02 cells transfected with indicated expression plasmids. HBx and Hdel (HBx with H-box deleted: AA88-98) were monitored by α-HA. (E) Percentage of pRPA32 IRIF-positive L02 cells pre-transfected with different HA-HBx constructs. pRPA32 foci were monitored 4 hours after irradiation. Cells with double positive staining of HA and pSerine 33 were judged as positive except in 'HBx$^-$' cells (no HA-HBx transfection) where pRPA32 were counted in random cells. Insert: co-staining of HA-HBx (green) and pSerine 33 (red). n = 3 biological repeats. Error bars = s.d. (F) IF for pRPA32 in L02 cells infected with HBx wild type or *R96E* lentivirus (2 MOI/cell) followed by IR treatment.

Fig.3. Disruption of $CRL4^{WDR70}$ complex by HBx results in a resection defect. (A) Sequence alignment for the H-boxes of cellular DCAF proteins and HBx (WDTC1: alias of DCAF9). Red triangle: binding hotspots serving as key DDB1-contacting residues. (B) By sequence alignment and secondary structure prediction, the WDR70 C-terminal sequence possesses similar H-box sequence and an adjunct α-helical structure to that of DDB2, indicating their similar docking mode to DDB1. (C) IP of endogenous DDB1 in 293T cells by Flag-tagged WDR70 with or without H-boxes (653-662 AA). V: empty vector; Hdel: H-box deletion mutant

of *WDR70*. (D) IF staining for phosphorylated RPA32 or RAD51 IRIF in parental and *WDR70* knockout (70KO) 293T cells with or without *HBx* expression. IRIF defects in *70KO* cells were not exacerbated by the presence of *HBx*. (E) Quantification for IRIF as in (D). n = 3 biological repeats. Error bars = s.d. NS: no significant difference. (F, G) Representative images and quantification for RAD51 (F) and pRPA32 (G) foci in IR-treated cells (4 Gy). Geminin-GFP represents S/G2 cells.

Fig.4. HBx influences $CRL4^{WDR70}$-dependent end resection via its H-box. (A) Immunoprecipitation of endogenous DDB1 by Flag-WDR70 in the presence, or not, of over-expressed HBx. Flag-WDR70 plasmid was transfected into L02 cells 24 hours before infection by pLV-HA-HBx lentivirus. Cells were lysed for co-IP analysis 48 hours after infection. α-WDR70 and α-DDB1 were used to detected individual proteins. (B) Immunoprecipitation of endogenous DDB1 by Flag-WDR70 in L02 and T43 cells. (C) Rescue of DDB1-WDR70 interaction in T43 cells by introducing *siHBx*. (D) Restoration of IR-induced pRPA32 by forced expression of DDB1 or Flag-WDR70 in *HBx* expressing L02 cells. Note that the protein level of HBx is elevated upon DDB1 over-expression, possibly due to a stabilising effect of their interaction (29). (E, F) Decreased RPA32 phosphorylation (E) and interaction between endogenous Flag-WDR70 and DDB1 (F) in L02 cells in the presence of HA-tagged SV5-V serving as an H-box surrogate for HBx.

Fig.5. HBx inhibits damage-induced H2B monoubiquitylation. (A-D) Evaluation of damage-induced H2B monoubiquitylation in *HB*x-expressing L02 cells (A), *WDR70*-(B) or *DDB1*-(C) depleted 293T cells and in PHHs with HBV replication (D). The densitometry of uH2B was determined relative to corresponding loading controls. H2B: loading control. (E) Immunoblotting for chromatin fractions of RPA32 probed with α-RAP32 serum in L02 cells with indicated treatments. Cells were treated with 2 uM CPT for 2 hours. H3: histone H3 level as loading control. (F) Partial restoration of H2B monoubiquitylation levels by over-expression of pLV-Flag-WDR70 in *HBx* expressing L02 (E). W+H: co-expression of Flag-WDR70 with HBx.

Fig.6. HBx impairs $CRL4^{WDR70}$-mediated extensive monoubiquitylation of H2B in the vicinity of DSBs. (A) Schematic representation for CRISPR-gRNA-DSB at human *PPP1R12C/p84* locus of chromosome 19. The gRNA targeting sequence in Intron 1 is highlighted in green. Upon transfection, CRISPR-RNA complexes digest DNA within a range of 100 bp downstream of the recognition site. Arrows, primer sets across cutting site; black bars, PCR amplicons for ChIP assays. The size of individual amplicons are 171 bp (0.5 Kb), 169 bp (3.5 Kb), 168 bp (10 Kb) and 162 bp (50 Kb), respectively. See also Supporting Fig. S7A-B. (B) Enrichment of H2B monoubiquitylation assayed by ChIP in *HBx*-expressing and *WDR70* knockout 293 cells, or in L02 and T43 cells. Distances from *PPP1R12C/p84*-specificCRISPR-gRNA (g1) induced DSBs were indicated at left. g0: control gRNA plasmid expressing no targeting sequence. (C, D) DSB-loading of RPA32 assayed by ChIP in

indicated cells and distance from breakpoint. (E, F) Chromatin loading of EXO1 (E) and MRE11 (F) assayed by ChIP in *HBx*-expressing and *WDR70* knockout 293T cells.

Fig.7. Loss of H2B monoubiquitylation promotes tumorigenesis. (A) Subcutaneous xenograft of L02 cells pre-infected by indicated lentivirus. Note the suppression of tumorigenesis of *2KR* by co-expression of *DDB1* or *WDR70*. (B) Immunoblotting for H2B monoubiquitylation in L02 cells by over-expression of human *USP22* (left). Cells expressing *USP22* were inoculated in nude mice and measured for tumor formation (right). (C) Xenograft tumor formation assay in nude mice. Individual tumor samples were dissected from nude nice 10 days after subcutaneous injection of L02 or T43 cells (left) and average tumor weight (right) were calculated. n = 9 dissected samples. Error bars: s.d. ** = $p<0.01$. (D) As in C, tumor formation assays for T43 cells pre-infected with pLV-vector, pLV-DDB1 or pLV-WDR70 lentivirus. (E) IHC images (inset) and quantification of uH2B positivity for paraffin-embedded xenografts. Nuclei were counterstained by hematoxylin (blue). n = 10 dissected samples.

Fig.8. Implication of H2B monoubiquitylation and resection defects in human HBVHCC. (A) Comparison of H2B monoubiquitylation by IHC in hepatocarcinoma with or without HBV infection. HBV carriers were diagnosed according to the ELISA test for serological positivity of surface antigen. HD/MD/PD: highly, moderately and poorly differentiated HCC. (B) Quantitative analysis by IRS for immunohistochemical staining of uH2B in HCC and non-HCC samples, see also Supplemental Methods. P=0.0057. (C) Impact on RPA32

phosphorylation and H2B monoubiquitylation in L02 cells by wild type and three clinically prevalent mutants of *HBx* at residuals 5, 130 and 131. None of the single, double or triple combination of mutations significantly diminishes the inhibitory effects of HBx on these cellular activities. (D) Model for the anti-resection activity of HBx on HR through $CRL4^{WDR70}$-dependent chromatin modification. Mirroring the deletion of yeast *Wdr70*, HBx dissembles the functional $CRL4^{WDR70}$ complex and results in decreased H2B monoubiquitylation, leading to an aberrant chromatin landscape. HBx impairs long-range resection. The resultant loss of homologous recombination promotes genome instability and thus provides a driver for the HBV-associated carcinogenesis.